AMENDMENTS TO THE SPECIFICATION:

Please replace the title with the following amended title:

INDUCTION OF TUBULAR MORPHOGENESIS USING PLEIOTROPHIN

Please delete Appendixes A and B. Applicants reserve the right to incorporate any essential matter in the application to support the claims as filed as presented in Appendixes A-B as filed.

Please replace paragraph [0001] on page 1 of the application with the following amended paragraph [0001]:

[0001] This application is a continuation-in-part and claims priority under 35 U.S.C. §119 to International Application Serial No. PCT/US02/20673, which International Application claims priority to U.S. Provisional Application Serial No. 60/301,684, filed June 28, 2001; this application also claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application Serial No. 60/426,152, filed November 14, 2002. ~~The following patent applications are related to and considered part of the application herein and are incorporated herein by reference in their entirety and are attached hereto as Appendices A and B: U.S. Provisional Application Serial Nos. 60/301,684 and 60/426,152, as well as U.S. Application Serial No. 09/965,651 and 09/595,195.~~

Please insert the following paragraphs between paragraphs [0037] and [0038] on page 8 of the application:

Figure 16: A schematic representation of the methodology and salient points of this invention. A schematic diagram showing a novel culture method for inducing *in vitro* branching morphogenesis of an isolated ureteric bud (UB), simultaneous culture of mesenchymal tissue and recombination and coculturing of the two cultured tissue fragments. The mesenchymal tissue added to the bud culture induces the bud to directionally extend branching tubules and further differentiate and incorporate to form a functioning nephron, capable of absorbing, filtering, collecting and secreting body fluids. Schematically depicted is a ureteric bud fragment in culture **2**, being induced by a stimulant(s) to produce a pluripotent

fragment **3**, that is capable of branching morphogenesis to form a branched three-dimensional structure **4**. It can be see that an excised growing tip **2** can be further cultured in the presence of an inducer(s) **1** to again form an activated fragment **3**, that will continue its tubulogenic morphogenesis. Simultaneously, an isolated fragment of mesenchymal tissue **5** is grown in culture to produce multiple pieces of mesenchymal tissue. One such piece **6** is grown and is then placed in coculture with an actively branching bud fragment **7**. The bud fragment, under influence of the mesenchymal induction continues to branch in a now directed fashion and to further differentiate to form maturing effluent collecting tubules, enlarging as the branching progresses to accommodate increased effluent and incorporating into new nephrons. Eventually an embryonic kidney, or a functionally equivalent fragment thereof, is formed **8**.

Figure 17: A novel culture system for *in vitro* branching morphogenesis of the ureteric bud (UB) UBs free from mesenchyme were micro-dissected from E-13 rat kidney rudiments and placed in an ECM gel suspension composed of type I collagen and growth factor-reduced Matrigel, and cultured in BSN cell-conditioned medium (BSN-CM) supplemented with 10% FCS and growth factors. Details are given elsewhere in the text. The cultured UB was monitored daily by microscopy.

Figure 18A-D: The UB undergoes branching morphogenesis *in vitro* and develops three-dimensional tubular structures in the absence of mesenchyme E-13 rat UB was isolated and cultured as described herein below. After culture, UBs were fixed at different time points and processed for DB lectin staining. 3-D reconstructions of confocal images are shown: a) A freshly isolated UB from an E-13 rat embryonic kidney with a single branched tubular structure; b) The very same UB shown in a) after being cultured for 3 days. The tissue has proliferated and small protrusions have formed; c) Again, the same UB as shown in a) cultured for 6 days. More protrusions have formed, and the protrusions have started to elongate and branch dichotomously; d) the same UB as shown in a) cultured for 12 days. The protrusions have undergone further elongation and repeated dichotomous branching to form a structure resembling the developing collecting system of a kidney. The white arrows indicate branch points. At higher power, the structures formed in this *in*

vitro culture system exhibited lumens. Phase microscopic examination and staining for markers revealed no evidence for contamination by other tissue or cells.

Figure 19A-D: BSN-CM and at least one soluble growth factor are required for branching morphogenesis of the isolated UB A: The UB cultured in the absence of BSN-CM and growth factors; B: The UB cultured with the mixture of growth factors (including EGF, IGF, HGF, FGF-2, and GDNF) but no BSN-CM; C: The UB cultured in the presence of BSN-CM alone; D: The UB cultured in the presence of both BSN-CM and the mixture of growth factors. All cultures were carried out for about one week and then processed for DB lectin staining. Shown is the three-dimensional reconstruction of confocal images. The isolated UB exhibits branching morphogenesis only in the presence of both BSN-CM and the mixture of growth factors.

Figure 20A-D: BSN-CM contains unique soluble factor(s) for branching morphogenesis of the isolated UB The UBs were cultured in the presence of the key growth factor (GDNF; see Fig. 22) but with different cell conditioned media: A: 3T3 fibroblast cell conditioned medium; B: immortalized UB cell conditioned medium; C: mIMCD cell conditioned medium; D: BSN cell conditioned medium. After culture, the UBs were fixed and processed for DB lectin staining. Only BSN-CM could promote extensive branching morphogenesis of the isolated UB.

Figure 21A-D: GDNF plus BSN-CM is required for branching morphogenesis The UBs were cultured in the presence of BSN-CM, as in Fig. 19 but with each of single growth factors present in the growth factor mixture. Several examples are shown: A: with EGF alone; B: with FGF-2 alone; C: with HGF alone; D: with GDNF alone. Only GDNF combined with BSN-CM could promote branching morphogenesis of the isolated UB.

Figure 22A-F: GDNF is required for both early and late branching morphogenesis *in vitro*. A-C: The antibodies against GDNF are neutralizing antibodies. A: UB was cultured in the presence of BSN-CM and GDNF without antibodies; B: same as A, but normal goat IgG antibody were added; C: same as A, but antibodies against GDNF were added. D-F: GDNF is required for branching morphogenesis. The UBs were initially cultured in the presence of BSN-CM and GDNF and then the cultures were washed to remove GDNF at different time points;

the UBs were then continuously cultured in BSN-CM without GDNF. To ensure neutralization of residual GDNF in the culture, antibodies against GDNF were added after removal and washing of GDNF from the culture medium. D: The UB was cultured as in A, but GDNF was removed and antibodies against GDNF were added on the first day of culture; E: Same as D, but the GDNF was removed and antibodies against GDNF were added on the second day of culture; F: Same as D, but the GDNF was removed and antibodies against GDNF were added on the third day of culture (compare with structures in Fig. 18). All cultures were carried out until the fifth day and processed with DB lectin staining. Whenever GDNF is depleted, UB growth and branching morphogenesis is aborted, indicating that GDNF is required for both early and late branching morphogenesis in vitro.

Figure 23A-I: The cultured three-dimensional tubular structure exhibits markers of UB epithelium and is functionally capable of inducing nephrogenesis when recombined with metanephric mesenchyme *in vitro*. The UBs were cultured in the presence of BSN-CM and GDNF and then stained for various markers (A-F). A: Light microscopic phase photograph of cultured UB; B: Staining with DB lectin, a ureteric bud specific lectin which binds to the UB and its derivatives; C: Staining for vimentin, a mesenchymal marker, D: Staining for N-CAM, the early marker for mesenchymal to epithelial conversion in the kidney, E: Staining with PNA lectin, a mesenchymally derived renal epithelial cell marker, F: Staining for cytokeratin, an epithelial marker. G-I: The cultured three-dimensional tubular structure is capable of inducing nephrogenesis when recombined with metanephric mesenchyme. The isolated UB was first cultured 7-10 days as shown in G. Then, the cultured UB was removed from the ECM gel and recombined with freshly isolated metanephric mesenchyme from E13 rat kidneys. The recombinant was cultured on a Transwell filter for another 5 days. After culture, the sample was double stained with DB lectin (FITC) and PNA lectin (TRITC) as shown in H and in the enlarged section of H shown in I. Results indicate that the in vitro cultured UB derived structures are capable of inducing nephrogenesis in vitro.

Figure 24A-B: Culture of metanephric mesenchyme. Day 13 embryonic rat kidneys rudiments were microdissected to separate the ureteric bud from the metanephric mesenchyme. The metanephric mesenchyme was then placed in a

Transwell tissue culture insert on top of the polycarbonate filter (3 μm pore size). Media (DME/F12) supplemented with 10% fetal calf serum (FCS) was placed in the bottom of the chamber and the entire setup was incubated at 37°C with 5% CO_2 with 100% humidity. (A) Freshly isolated metanephric mesenchyme. (B) The same metanephric mesenchyme following 5 days in culture.

Figure 25A-D: Subculture of the ureteric bud. Ureteric buds were isolated from E13 rat kidneys and grown in culture for 7 days. At the end of this culture period the ureteric bud was dissected free of the surrounding extracellular matrix and the bud was cut into pieces and subcultured under the same conditions. (A) Originally isolated ureteric bud after seven days of culture. Black box indicates piece of bud that was dissected free and subcultured. (B) Subcultured bud after 24 hrs in culture. (C) Subcultured bud after 4 days in culture. (D) Subcultured bud after 7 days in culture.

Figure 26: Recombination of subcultured bud with freshly isolated metanephric mesenchyme. Ureteric buds were isolated, cultured and subcultured as previously described in Fig. 25. Metanephric mesenchymes were microdissected from E13 day rat embryonic kidneys and placed in close contact with subcultured ureteric bud as in Fig. 23. The recombined tissues were grown in culture for 7 days. Tubular structures are evident at this time.

Please insert the following paragraphs between paragraphs [00159] and [00160] on page 47 of the application:

Example 26

Isolation of ureteric bud (UB) epithelium and UB culture Kidney rudiments were dissected from timed pregnant Sprague Dawley rats at gestation day 13. (The plug day was designated as day 0). The UB was isolated from mesenchyme by incubating kidney rudiments in 0.1% trypsin in the presence of 50 U/ml DNAase at 37°C for 15 minutes, and by mechanical separation with two fine-tipped minutia pins. For culture, Transwell tissue culture plates and a polycarbonate membrane insert with 3 um pore size were used. The extracellular matrix (ECM) gel (a mixture of type I collagen and Matrigel) was applied on top of the Transwell insert. Isolated UB was suspended in the ECM gel and cultured at the interface of air and

medium. All cultures were carried out at 37°C with 5% CO_2 and 100% humidity in DMEM/F12 supplemented with 10% Fetal Calf Serum (FCS). Growth factors were added as indicated elsewhere. Culture media were changed weekly if necessary.

Example 27

Cells and conditioned media: The BSN cell line was derived from day 11.5 mouse embryonic kidney metanephric mesenchyme originally obtained from a mouse line transgenic for the early region of SV-40/large T antigen. As described elsewhere, the BSN cells express the mesenchymal protein marker vimentin, but not classic epithelial marker proteins such as cytokeratin, ZO-1 and E-cadherin. Differences in the expression patterns of 588 genes in BSN cells have been analyzed by the inventors on commercially available cDNA grids (Am. J. Physiol.-Renal Physiol., 277, F:650-F663, 1999), and confirmed the largely non-epithelial character of BSN-cells, though it remains to be determined whether they 10 are mesenchymal or stromal, or have characteristics of both cell types. The SV-40/large T antigen transformed UB cell line and routine inner-medulla collecting duct (mIMCD) cells have been extensively characterized before. To obtain conditioned media, a confluent cell monolayer was washed with serum-free medium, and then cultured in serum free medium for another 2-4 days.

Various conditioned media were harvested after low speed centrifugation to remove cell debris and then concentrated 10-fold with a Centricon filter with 8 kDa nominal molecular weight cutoff (Millipore, Bedford, MA). In addition, BSN-CM was subfractionated on a heparin-sepharose affinity column (Hitrap Heparin; Pharmacia, NJ). Concentrated BSN-CM (~10X) was applied to a heparin column. After washing the column with Hanks' balanced buffer solution, the heparin bound fraction was eluted with 2 M NaCl in Hanks' balanced buffer solution. After desalting with a PD-10 column (Pharmacia, NJ), the heparin bound fraction's final volume was adjusted to the starting volume. The heparin flow through fraction was collected and its volume was adjusted to the starting volume using a Centricon filter (8 kDa cutoff). The partially purified fractions were assayed for their effect on UB morphogenesis in the presence of GDNF.

Example 28

The ECM gel mix: The ECM gel mix was composed of 50% type I collagen (Collaborative Biomedical Product) and 50% growth factor-reduced Matrigel (Collaborative Biomedical Product). The procedure for gelation has been previously described in detail and is incorporated herein.

Example 29

Induction of nephrogenesis by cultured UB: Isolated UBs were first cultured for 7-10 days as already described. Then, the cultured UB was isolated from the ECM gel by incubation with collagenase (1 mg/m1) and dispase (2 ml/ml) at 37°C for 30 minutes, followed by mechanical separation with fine tipped minutia pins. The UB was then recombined with freshly isolated E-13 rat metanephric mesenchyme and co-cultured on a transfilter for another 5 days in DMEM/F12, plus 10% FCS.

Example 30

Lectin staining: 1) Dolichos Biflorus (DB) lectin: Tissues were fixed with 2% paraformaldehyde for 30 minutes at 4°C, permeabilized with 0.1% Saponin and then incubated with fluorescent conjugated DB (50 ug/ml, Vector) in a moisturized chamber for 60 minutes at 37°C. After extensive washing, tissues were post-fixed in 2% paraformaldehyde again for 5 minutes and viewed using a laser scanning confocal microscope. The specificity of DB lectin binding has been demonstrated previously. 2) Peanut agglutinin (PNA) lectin: Tissues were fixed with 2% paraformaldehyde for 30 minutes at 4°C; blocked with 50 mM NH_4Cl overnight at 4°C, followed by an incubation with 1% gelatin in 0.075% Saponin for 30 minutes at 37°C. After two washes with Neuraminidase buffer (150 mM NaCl, 50mM Na-Acetate, pH 5.5), tissues were incubated with Neuraminidase (1 U/ml) for 4 hours at 37°C and then with Rhodamine-conjugated PNA (50 ug/ml) for 60 minutes at 37°C. Tissues were post-fixed with 2% paraformaldehyde and viewed with a laser scanning confocal microscope.

Example 31

Immunocytochemistry: Tissues were fixed with either 2% paraformaldehyde at 4°C or 100% methanol at -20°C. Tissues were permeablized with 0.1% Saponin and non-specific binding was blocked with fetal 100% FCS. The incubations with primary and secondary antibodies were carried out for 60 minutes at 37°C. The staining with FITC or TRITC-conjugated antibodies was viewed with a laser scanning confocal microscope.

Example 32

Confocal Analysis: Confocal images were collected with a laser scanning confocal microscope (Bio-Rad MRC 1024, Bio-Rad, CA). Each three-dimensional picture was reconstructed from a set of 10 um serial sections, which spanned the tissue. Images were processed with Laser Sharp™ (Bio-Rad) and Photoshop™ (Adobe, CA) software.

Please renumber the paragraphs of the specification accordingly.